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# *Bacillus cereus* Group-Type Strain-Specific Diagnostic Peptides

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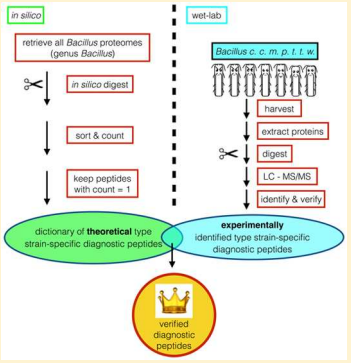
Supporting Information

**ABSTRACT:** The *Bacillus cereus* group consists of eight very closely related species and comprises both harmless and human pathogenic species such as *Bacillus anthracis, Bacillus cereus*, and *Bacillus cytotoxicus*. Numerous efforts have been undertaken to allow presumptive differentiation of *B. cereus* group species from one another. However, methods to rapidly and accurately distinguish these species are currently lacking. We confirmed that classical matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) biotyping cannot achieve reliable identification of each type strain. We therefore assigned type strain-specific diagnostic peptides to the *B. cereus* group based on comparisons of their proteomic profiles. The number of diagnostic peptides varied remarkably in a type strain-dependent manner. The accuracy of the reference database was crucial to validate candidate diagnostic peptides and led to a noteworthy reduction of verified diagnostic peptides. Diagnostic peptides ranged from one for *B. weihenstephanensis* to 62 for *B. pseudomycoides* and were associated with proteins involved in diverse biological processes, e.g. amino acid biosynthesis, cell envelope, cellular processes, energy metabolism, and transport processes. However, 45.6% of all diagnostic peptides comprised currently unclassified proteins or proteins of unknown function. In addition, a phylogenetic tree based on clustering of theoretical precursor masses deduced from *in silico*-generated tryptic peptides was reconstructed.




**KEYWORDS:** *Bacillus cereus group, type strains, diagnostic peptides, mass spectrometry*

## INTRODUCTION

The *Bacillus cereus* group species are Gram-positive, endospore-forming bacteria, which comprise *B. anthracis*, *B. cereus*, *B. cytotoxicus*, *B. mycoides*, *B. pseudomycoides*, *B. thuringiensis*, *B. toyonensis*, and *B. weihenstephanensis*.[1−4] Despite low genetic diversity,[5−7] they differ remarkably in their medical and public health importance.[8,9] Methods to rapidly and accurately differentiate these species are currently lacking. Hence, the fields of clinical, environmental, and food microbiology, as well as biodefense would benefit from a reliable, rapid, and efficient technique to differentiate these species.

*Bacillus cereus* (*sensu stricto*) exists ubiquitously in nature and acts as an opportunistic pathogen.[10−12] This species is associated with food poisoning by causing either emetic or diarrheal syndromes due to the ingestion of the cyclic and heat-stable toxin cereulide or the production of enterotoxins.[13−16] Guinebretière et al. (2013)[2] have classified *B. cytotoxicus* as a novel species of the *B. cereus* group and described its occasional association with food poisoning. *Bacillus cereus* (*sensu stricto*) and *B. cytotoxicus* pose a potential threat to the public health via food industry and agriculture, as contamination with their spores or vegetative cells may (I) harm the consumers' health, in worst case even lead to fatalities,[2,17] (II) result in lost revenue, and (III) damage reputation. *Bacillus anthracis* can be detrimental to both animal and human health and has been abused in bioterrorism.[18] Infected people may develop different disease forms of anthrax (cutaneous, gastrointestinal, and inhalational), which can be fatal if untreated.[8,19] On the contrary, *B. thuringiensis* received attention due to its ability to produce crystalline toxins exhibiting insecticidal activity.[20] Accordingly, this microorganism has industrial application as a biological pesticide and its molecular genetics served as a basis for the bioengineering of transgenic plants and for plant-associated bacteria such as *Pseudomonas fluorescens*.[21,22] However, certain *B. thuringiensis* isolates have also been linked to human cutaneous infections.[23] *Bacillus weihenstephanensis* can grow below 7 °C[24,25] and occasionally encodes the emetic toxin cereulide.[26,27] The combination of these two characteristics may pose a problem from a food safety perspective. The remaining *B. cereus* group species, that is, *B. mycoides*, *B. pseudomycoides*, and *B. toyonensis*, are considered to be nonpathogenic organisms.[24,28,29] *Bacillus mycoides* and *B. pseudomycoides* are easily recognized by their rhizoidal colonial growth,[28,29] whereas they differ from each other in their fatty acid profiles.[29] *Bacillus toyonensis* has formerly been used as probiotic strain in animal nutrition (for a detailed historical report see Jiménez, Uridain et al. (2013)[3]).

Numerous molecular studies investigating whole-genome DNA hybridization,[30] sequences of 16S and 23S rRNA genes as

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well as single nucleotide polymorphisms (SNPs) in the intergenic 16S-23S rRNA regions,[31−34] pulsed-field gel electrophoresis (PFGE),[35] multilocus enzyme electrophoresis,[6] genomic mapping and rep-PCR fingerprinting,[36,37] multilocus sequence typing (MLST),[5,38] and amplified fragment length polymorphism (AFLP)[39] have revealed pronounced genomic similarities. Various biochemical assays and immunoassays allow presumptive differentiation of *B. cereus* group species from one another.[8] However, these conventional molecular and immunological-based identification and classification techniques are time-consuming and expensive, and require a combination of several tests. Matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) biotyping is a valuable and well-established technique to rapidly identify bacterial species.[40−43] As the members of the *B. cereus* group are genetically very similar,[5] MALDI biotyping results are reliable on genus but not on species level.[44] Furthermore, mass spectra quality depends on the physiological state of the bacilli as progression of sporulation; that is, the presence of spores, can suppress several of the few characteristic mass ions.[45,46]

The objective of this study was to assign type strain-specific diagnostic peptides to the various members of the *B. cereus* group. We analyzed *in silico*-generated tryptic peptides to estimate the potential number of type strain-specific diagnostic peptides and reconstructed a phylogenetic tree based on the clustering of theoretical precursor masses. We applied a rapid cell extraction procedure[47] combined with fast generation of tryptic peptides using high-intensity focused ultrasound (HIFU)[48,49] to obtain trypsin-digested cell extracts. HIFU improves the solubilization of proteins[50] and enables proteolytic digestion within 15 min. Proteomes were analyzed using liquid chromatography electrospray ionization tandem mass spectrometry (LC−ESI MS/MS). We were limited in analyzing *B. anthracis* digests as our institute does not have the infrastructure appropriate for biosafety level 3 facilities. The relevant quantity and quality of entries, including the correct classification of microorganisms, available on public databases are crucial for the present study. We verified the nominated candidate peptides twice within the theoretical background provided by the entries on Universal Protein Resource (UniProt, www.uniprot.org, status of May and October 2015),[51] because within six months, the addition of new entries on UniProtKB challenged the theoretical uniqueness of diagnostic peptides mainly from *B. toyonensis*. Comparing the number of candidate diagnostic peptides with the number of verified diagnostic peptides revealed that a remarkable decrease ranging from 26.3% for *B. cytotoxicus* to 99.6% for *B. weihenstephanensis* occurred.

## MATERIALS AND METHODS

### Bacterial Strains and Culture Conditions

Seven type strains of the *B. cereus* group were investigated. *Bacillus cereus* (DSM 31), *B. cytotoxicus* (DSM 22905), *B. mycoides* (DSM 2048), *B. pseudomycoides* (DSM 12442), *B. thuringiensis* (DSM 2046), and *B. weihenstephanensis* (DSM 11821) were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). *Bacillus toyonensis* (CECT 876) was kindly provided by Guillermo Jiménez.[3] All strains were grown aerobically in nutrient broth (5 g/L peptone from soymeal (Merck, Darmstadt, Germany), 3 g/L meat extract (Sigma-Aldrich, St. Louis, USA), adjusted to pH 7.0 with NaOH) for approximately 16 h at 30 °C or at 37 or 25 °C for *B. cytotoxicus* and *B. mycoides*, respectively. To ensure homogeneous suspension of *B. mycoides* and *B. pseudomycoides* in liquid cultures, cells were cultured in baffled 250 mL-flasks (Schott Duran, Mainz, Germany) containing 30 mL nutrient broth media. The other strains were grown in 5 mL nutrient broth media at the conditions mentioned above at 220 rpm.

### Analysis of in Silico-Generated Tryptic Peptides

Proteomes from the eight type strains of the *B. cereus* group available on Universal Protein Resource (UniProt, www.uniprot.org) (status of September 2015) were *in silico* analyzed using R (version 3.1.2).[52] Tryptic peptides (without missed cleavages) and their corresponding peptide masses were generated using the R language with some additional packages such as "cleaver" from bioconducter repository,[53] "SeqInR",[54] and "protViz"[55] from CRAN library. The heat map with dendrograms for the hierarchical clustering based on accurate theoretical precursor masses for *in silico*-generated tryptic peptides was created using the "gplots" package from CRAN library.[56]

### Identification of Diagnostic Peptides and Classical Biotyping

All chemicals were of analytical grade and were purchased from either Sigma-Aldrich (St. Louis, USA), Biosolve (Valkenswaard, Netherlands), or Merck (Darmstadt, Germany) if not indicated otherwise.

### Protein Extraction

For each type strain, two technical replicates of three independent biological replicates containing approximately $10^8$ CFU/mL were prepared by ethanol/formic acid extraction according to the Bruker biotyping protocol.[47] Briefly, 1 mL of bacterial overnight culture (adjusted to approximately $10^8$ CFU/mL) was collected at 5000*g* for 5 min and washed once with 1 mL of Milli-Q water. The pellet was resuspended in 75% ethanol (EtOH) and centrifuged at 16100*g* for 2 min. EtOH was discarded and any residual solution was evaporated at room temperature for 5−10 min. The pellet was mixed thoroughly with 100 μL of 70% formic acid (FA) to disrupt the cell walls. The sample was incubated for at least 5 min in FA before the addition of 100 μL of acetonitrile (ACN). The protein-containing supernatant was separated from the cell debris and used for (I) classical biotyping and (II) identification of diagnostic peptides.

### MALDI-TOF Measurement

Each extract (1 μL) was spotted in triplicates onto a MALDI target (MSP 96 polished steel BC target; Bruker Daltonics, Bremen, Germany). After drying, the sample spots were covered with 1 μL of α-cyano-4-hydroxycinamic acid (Bruker Daltonics) solution at a concentration of 10 mg/mL in ACN−water−trifluoroacetic acid (50:47.5:2.5 [vol/vol/vol]). MALDI mass spectra were acquired using a microflex LT MALDI-TOF mass spectrometer (Bruker Daltonics). The following instrument parameters were chosen as suggested by the manufacturer and as described in ref 48: laser frequency of 60 Hz in the positive linear mode with an acquisition range of $m/z$ = 2000 to 20000. Final spectra represented the sum of 240 shots per spot (40 shots per raster spot and random laser movement) using a laser intensity of approximately 40−50% with a global laser attenuator offset of 22% resulting in maximal absolute peak intensities between (3−6) × $10^4$ arbitrary units. Classification was performed using the Realtime Classification Biotyper


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software (version 3.1) (Bruker Daltonics) containing 5627 MSPs in the MALDI Biotyper DB (V4.0.0.1).

### Trypsin Digestion

To identify type strain-specific diagnostic peptides, the residual protein-containing supernatants (190 μL) were evaporated to dryness using a SpeedVac concentrator (Savant, Thermo Scientific, San Jose, USA). Proteins were resolubilized in 5 μL of ACN and 40 μL of Tris-$CaCl_2$ (10 mM, 2 mM, pH = 8.2) with assistance of high-intensity focused ultrasound (HIFU, UTR200 reactor, Hielscher, Teltow, Germany) for 5 min, at 75% intensity, 0.6 s cycle time, and a final water temperature of 26−29 °C. Five μL of 100 ng/μL trypsin (Roche Diagnostics, Mannheim, Germany) in 10 mM HCl were added to digest the proteins using HIFU assistance for 15 min, 75% intensity, 0.6 s cycle time, and a final water temperature of 38−40 °C. The digestion reaction was stopped by addition of 2 μL of 1 M HCl. Samples were evaporated to dryness with a SpeedVac concentrator and stored at −20 °C until further analysis.

### LC−MS/MS Analysis

Digested samples were resolubilized in 20 μL of 0.1% FA with assistance of an ultrasound bath for 1 min and thorough vortexing and were analyzed on a nanoAcquity UPLC (Waters Inc., Milford, USA) connected to a Q Exactive mass spectrometer (Thermo Scientific) equipped with a Digital PicoView source (New Objective, Woburn, USA). An aliquot of 2 μL was injected. Peptides were trapped on a Symmetry C18 trap column (5 μm, 180 μm × 20 mm, Waters, Inc.) and separated on a BEH300 C18 column (1.7 μm, 75 μm × 150 mm, Waters Inc.) at a flow rate of 250 nL/min using a gradient from 1% solvent B (0.1% FA in ACN)/99% solvent A (0.1% FA in water) to 40% solvent B/60% solvent A within 90 min. Full scan MS spectra were acquired in positive profile mode from $m/z$ 350 to $m/z$ 1500 with an automatic gain control target of $3 \times 10^6$, an Orbitrap resolution of 70000 (at $m/z$ 200), and a maximum injection time of 100 ms. The 12 most intense multiply charged ($z \geq +2$) precursor ions from each full scan were selected for higher-energy collisional dissociation (HCD) with a normalized collision energy of 25 (arbitrary unit). Generated fragment ions were scanned with an Orbitrap resolution of 35000 (at $m/z$ 200), an automatic gain control value of $1 \times 10^5$, and a maximum injection time of 120 ms. The isolation window for precursor ions was set to $m/z$ 2.0 and the underfill ratio was at 3.5% (referring to an intensity threshold of $2.9 \times 10^4$). Each fragmented precursor ion was set onto the dynamic exclusion list for 40 s.

### Data Analysis

Peak lists in Mascot generic file (MGF) format were generated using Proteome Discoverer (v1.4) by picking the 10 most intense peaks/100 Da and the MGF files were generated using the FGCZ Converter Control.[57] These were searched against a *Bacillus*-specific database which is composed of 113 bacilli proteomes (forward and reversed for target-decoy false discovery rate (FDR) estimation) with 1158177 bacilli protein sequences (Supplementary Table S-1) available on UniProt (www.uniprot.org, status of May 2015) using Mascot search engine (version 2.4.1, Matrix Science Ltd., London, UK). The search was restricted to tryptic peptides with variable methionine oxidation and variable asparagine and glutamine deamidation, precursor mass tolerance of 10 ppm, fragment mass tolerance of ±0.04 Da, and maximum one missed cleavage. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository[58] with the data set identifier PXD003669 and 10.6019/PXD003669.

### Identification of Candidates and Verification of Diagnostic Peptides

Proteomics data were validated using Scaffold software (version 4.4.1.1, Proteome Software, Portland, USA) with the following settings: protein threshold at 2.0% false discovery rate (FDR); peptide threshold at 1.0% FDR; and two peptides minimum. The peptide report was exported and candidate peptides were selected by creating a pivot table in Microsoft Excel 2013 (Supplementary Table S-2). Peptides were nominated as candidates if they (I) did not contain any missed cleavages, (II) occurred in all three biosamples of a type strain, and (III) were not detected in any sample of the other six type strains examined. Candidates were verified using R (version 3.2.1) by confirming that the diagnostic peptide sequences were uniquely present in one particular *Bacillus* species and absent in all the other *Bacillus* species listed in the above-mentioned *Bacillus*-specific database by string comparison. By October, new entries on UniProt demanded a second validation step. Candidate peptides were blasted against the UniProtKB database using the following parameters: expectation value threshold of 0.0001; auto assignment of the matrix; no filtering; no gaps; 1000 hits. If no hits were found for the query, previously assigned protein identities were allowed to retrieve and blast the entire protein sequence data in FASTA format against the UniProtKB database using the search parameters mentioned above.

## RESULTS

### Determination of *Bacillus cereus* Group Type Strain-Specific Diagnostic Peptides

To identify type strain-specific diagnostic peptides of the *B. cereus* group (*B. cereus*, *B. cytotoxicus*, *B. mycoides*, *B. pseudomycoides*, *B. thuringiensis*, *B. toyonensis*, and *B. weihenstephanensis*), we extracted proteins from 1 mL overnight liquid cultures containing approximately $10^8$ CFU/mL, digested the extracts with trypsin using HIFU, and analyzed the proteolytic samples with a Q Exactive Hybrid Quadrupole-Orbitrap mass spectrometer. Three independent biological replicates were analyzed for each type strain. Experimentally unique peptides were nominated as candidate diagnostic peptides if they (I) did not contain any missed cleavages, (II) occurred in all three independent replicates of a type strain, and (III) were not detected in any of the other type strains investigated (Table 1). A total of 6129 individual proteins were detected at least once among all type strains and replicates. A list of all proteins identified is provided in the Supporting Information Table S-2 (protein name, accession number, identification probability, sequence coverage, and other relevant information), where candidate diagnostic peptides are highlighted in light gray. Table S-2 also summarizes information on peptide identification (sequence match, Mascot scores, peptide identification probability, and charge).

Candidate diagnostic peptides were blasted against a *Bacillus*-specific database composed of 113 proteomes downloaded from Universal Protein Resource (UniProt, www.uniprot.org) in May 2015 (consult Supporting Information Table S-1 for an overview of the proteomes downloaded). If the sequence of candidate diagnostic peptide was not present in any theoretical proteome of another *Bacillus* species, its validity was accepted.


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Table 1. Number of Candidate and Verified Diagnostic Peptides for Type Strains of the *B. cereus* Group[a]

| species | number of candidate diagnostic peptides (detected as experimentally unique) | verified diagnostic peptides (UniProtKB, status May 2015) | verified diagnostic peptides (UniProtKB, status October 2015) |
|---|---|---|---|
| *B. ce.* | 87 | 6 (6.9%) | 3 (3.4%) |
| *B. cy.* | 19 | 17 (89.5%) | 14 (73.7%) |
| *B. my.* | 540 | 73 (13.5%) | 4 (0.7%) |
| *B. ps.* | 1309 | 81 (6.2%) | 62 (4.7%) |
| *B. th.* | 382 | 174 (45.5%) | 58 (15.2%) |
| *B. to.* | 67 | 9 (13.4%) | 7 (10.4%) |
| *B. we.* | 266 | 2 (0.8%) | 1 (0.4%) |

[a]Notation: *B. ce.*, *B. cereus*; *B. cy.*, *B. cytotoxicus*; *B. my.*, *B. mycoides*; *B. ps.*, *B. pseudomycoides*; *B. th.*, *B. thuringiensis*; *B. to.*, *B. toyonensis*; *B. we.*, *B. weihenstephanensis*.

Blasting was performed at two different time points, May and October 2015, resulting in a noteworthy reduction of the absolute number of validated diagnostic peptides. The values 3.4%, 73.7%, 0.7%, 4.8%, 15.2%, 10.4%, and 0.4% denote the ratios of the number of verified diagnostic peptides versus the number of candidate diagnostic peptides for *B. cereus*, *B. cytotoxicus*, *B. mycoides*, *B. pseudomycoides*, *B. thuringiensis*, *B. toyonensis*, and *B. weihenstephanensis*, respectively (Table 1). New entries on UniProt Knowledgebase, predominately the addition of newly submitted proteomes from putative *B. cereus* strains, challenged the theoretical uniqueness of the verified diagnostic peptides from *B. toyonensis*. Böhm et al. (2015)[7] suggested to review the affiliations of certain *Bacillus* strains as their classification did not match the genomic relationships presented in their study. Böhm and co-workers[7] developed phylogenetic networks based on average nucleotide identity (ANI) from genome sequences and whole-genome SNP analysis subdividing *B. cereus sensu lato* into seven phylogenetic groups. These authors suggested renaming all isolates belonging to the phylogenetic group V as *B. toyonensis* species, which allows the validation of seven from the nine diagnostic peptides assigned to *B. toyonensis* in May 2015 (Table 1 and Table S-3).

All diagnostic peptides validated in October 2015 are listed in Table S-3, whereas Table 2 groups the associated proteins containing at least one diagnostic peptide according to their TIGR (The Institute for Genomic Research) function roles. Figure 1 depicts the workflow of our binary approach to identify type strain-specific diagnostic peptides.

### MALDI-TOF Analysis (Classical Biotyping)

Before LC−MS/MS analysis, MALDI-TOF MS mass spectra were obtained for each cell extract in triplicates. Certain type strains of the *B. cereus* group cannot reliably be distinguished using classical MALDI-TOF MS biotyping based on the Bruker Biotyper software (version 3.1) (Table 3). The number of proteins detected by classical MALDI biotyping ranged from 40 to 54 in a mass range of $m/z$ = 3000 to 20000. By performing LC−MS/MS analysis, about 45 to 80 times more proteins could be detected.

### Phylogenetic Tree Reconstruction Based on Analysis of Reference Proteomes of the Type Strains from the *B. cereus* Group

We predicted the number of putative unique tryptic peptides *in silico* due to the low genetic diversity of the species belonging to the *B. cereus* group[5] and technical limitations in the analysis of *B. anthracis* (Table 4). We only considered mass spectrometry compatible peptides in a mass range of 500 to 6000 Da that did not contain the amino acids methionine or cysteine. A list of all *in silico*-generated candidate diagnostic peptides is provided in Supporting Information Table S-4. Theoretical tryptic peptides of reference proteomes from the eight type strains available on UniProt were analyzed using R language. We reconstructed a phylogenetic tree based on theoretical precursor masses (Figure 2).

Table 2. Proteins Containing Verified Diagnostic Peptides from Type Strains of the *B. cereus* Group Grouped According to TIGR Role Categories[a]

| TIGR function roles | *B. ce.* | *B. cy.* | *B. my.* | *B. ps.* | *B. th.* | *B. to.* | *B. we.* |
|---|---|---|---|---|---|---|---|
| amino acid biosynthesis | | | | 5 | 1 | | |
| biosynthesis of cofactors, prosthetic groups and carriers | | | | 1 | | | |
| cell envelope | 1 | 3 | | 1 | 2 | | |
| cellular functions | | | | | | 1 | |
| cellular processes | 1 | 2 | | 5 | 11 | | |
| DNA metabolism | | | | 2 | 1 | 1 | |
| energy metabolism | | 1 | | 5 | 4 | 2 | |
| fatty acid and phospholipid metabolism | | | | 3 | | | |
| hypothetical proteins | | 1 | | | | | |
| protein fate | | | | 3 | 1 | 1 | |
| protein synthesis | | 1 | | 4 | | | |
| purines, pyrimidines, nucleosides, and nucleotides | | 1 | 1 | 1 | | | |
| regulatory functions | | | | 2 | 2 | | 1 |
| transcription | | | | | 1 | | |
| transport and binding proteins | 1 | | | 6 | 1 | | |
| unclassified | | 2 | 2 | 21 | 26 | 1 | |
| unknown function | | 3 | 1 | 3 | 8 | 1 | |
| total | 3 | 14 | 4 | 62 | 58 | 7 | 1 |

[a]Notation: *B. ce.*, *B. cereus*; *B. cy.*, *B. cytotoxicus*; *B. my.*, *B. mycoides*; *B. ps.*, *B. pseudomycoides*; *B. th.*, *B. thuringiensis*; *B. to.*, *B. toyonensis*; *B. we.*, *B. weihenstephanensis*.

## DISCUSSION

*Bacillus cereus* group members are genetically very closely related,[5−7] however they differ remarkably in terms of their medical and public health implications.[8] In this study, we have identified type strain-specific diagnostic peptides to provide the basis for the development of a diagnostic tool to differentiate *B. cereus* group species. The diagnostic peptides are associated with various proteins involved in diverse biological processes. Grouping of the associated proteins according to TIGR role categories exemplifies the large variety of those processes (Table 2). The categories cell envelope, cellular processes, energy metabolism, unclassified, and unknown function are covered by at least four of the seven type strains investigated. The variation of the number of verified diagnostic peptides per type strain may be explained by (I) the very high genetic relatedness of distinct *B. cereus* group strains combined with their different genome sizes; for example, *B. cytotoxicus* comprises ∼3840 proteins, which is ∼$^1/_4$ less than the other type strains, (II) the number of sequenced genomes available per *B. cereus* species, (III) not exploiting specific phenotypic


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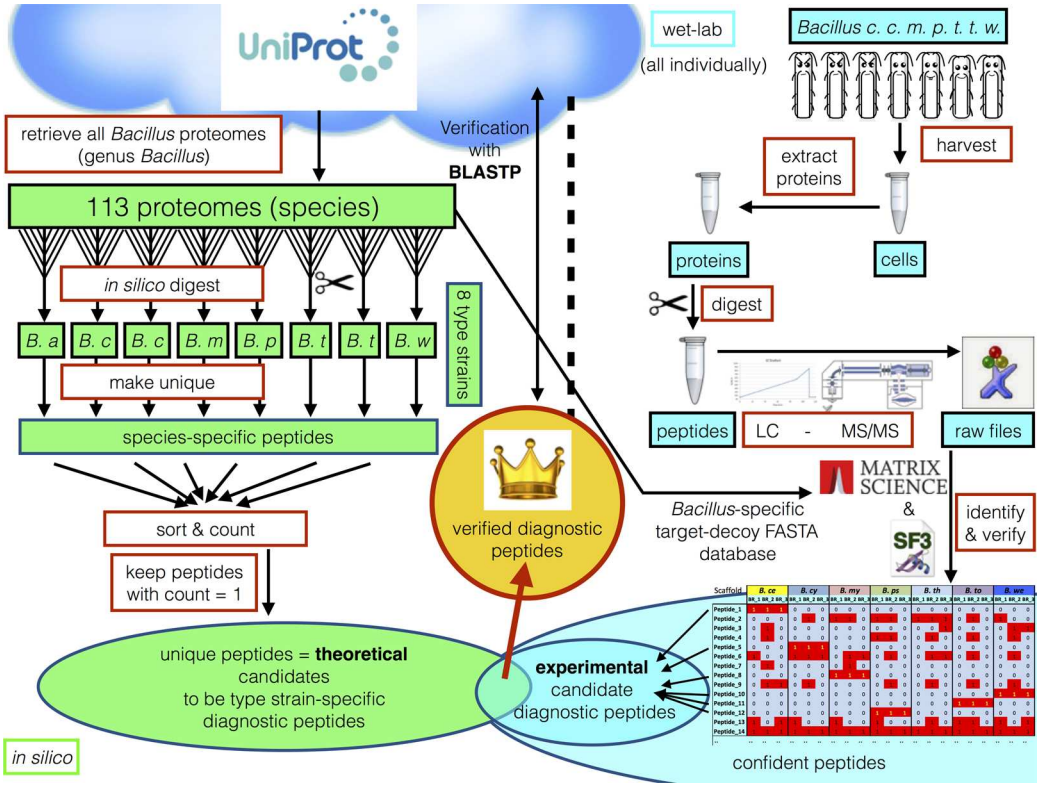


**Figure 1.** Workflow depicting our main approaches, that is, *in silico* (green) and wet-lab (blue), to identify *B. cereus* group type strain-specific diagnostic peptides.

**Table 3. Comparison of Average Identification Scoring Values for *B. cereus* Group Members Using Classical Biotyping[a]**

| species | average score (best match) | SD | best match |
|---|---|---|---|
| *B. an.* | | | |
| *B. ce.* | 2.384 | 0.036 | 18/18 *Bacillus cereus* DSM 31T DSM |
| *B. cy.* | 1.848[b] | 0.050 | 17/18 *Bacillus cereus* (DSM 31T DSM or 994000168 LBK) 1/18 *Bacillus thuringiensis* DSM 2046T DSM |
| *B. my.* | 2.413 | 0.045 | 18/18 *Bacillus mycoides* DSM 2048T DSM (Second best match: 18/18 *Bacillus weihenstephanensis* DSM 11821T, Average score: 2.131 ± 0.063) |
| *B. ps.* | 2.113 | 0.036 | 18/18 *Bacillus pseudomycoides* DSM 12442T DSM |
| *B. th.* | 2.102 | 0.077 | 16/18 *Bacillus thuringiensis* DSM 2046T DSM 2/18 *Bacillus cereus* DSM 31T DSM (Average score: 2.065 ± 0.027) |
| *B. to.* | 2.186[b] | 0.050 | 17/18 *Bacillus cereus* DSM 31T DSM (Average score: 2.191 ± 0.046) 1/18 *Bacillus thuringiensis* DSM 2046T DSM (score: 2.101) |
| *B. we.* | 2.210 | 0.112 | 8/18 *Bacillus weihenstephanensis* DSM 11821T DSM 9/18 *Bacillus mycoides* DSM 2048T DSM (Average score: 2.211 ± 0.168) 1/18 *Bacillus cereus* DSM 31T DSM (Score: 1.841) |

[a]Note: $n = 3$ independent replicates (prepared in two technical replicates each) were analyzed and spotted in triplicates. SD = standard deviation. *B. an.*, *B. anthracis*; *B. ce.*, *B. cereus*; *B. cy.*, *B. cytotoxicus*; *B. my.*, *B. mycoides*; *B. ps.*, *B. pseudomycoides*; *B. th.*, *B. thuringiensis*; *B. to.*, *B. toyonensis*; *B. we.*, *B. weihenstephanensis*. [b]No entry in current Bruker Taxonomy Database (MALDI Biotyper DB V4.0.0.1).

characteristics during strain cultivation, that is, psychrotolerance (*B. weihenstephanensis*), rhizoidal growth (*B. mycoides* and *B. pseudomycoides*), thermotolerance (*B. cytotoxicus*), which could result in a differential protein expression profile,[25] and (IV) probable incorrect affiliation of individual strains[7] deposited on UniProt. Rarely, more than one diagnostic peptide is found within the same protein. These rare cases are linked to an ABC transporter (*B. pseudomycoides*), aromatic amino acid decarboxylase (*B. pseudomycoides*), nonribosomal peptide synthetase C (*B. pseudomycoides*), cell wall hydrolase (*B. cytotoxicus*), and flagellin (*B. thuringiensis*) and make up 14.1% of all verified diagnostic peptides (Table S-3). A large number of verified diagnostic peptides (45.6%) are associated with unclassified proteins or proteins of unknown function. Verified diagnostic peptides were not found within homologous proteins of the different type strains with one exception: *B. pseudomycoides* as well as *B. thuringiensis* have verified diagnostic peptides that are associated with flagellin (Table S-3). Only one diagnostic peptide was identified for *B. weihenstephanensis* (Table S-3). We may have lost candidate diagnostic peptides because we did not grow the cultures at low temperatures and thus did not exploit a temperature-specific protein expression profile of this psychrotolerant species.[25] Instead, we chose comparable culturing conditions for all type strains to facilitate the development of a simple assay to differentiate isolates from the *B. cereus* group. It is worth mentioning that cells which have transformed from the vegetative to the sporulative state would introduce additional signals into the mass spectral protein and peptide profiles. Rapid detection of spores of the respective species is important, for example, in the investigation of


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**Table 4. Predicted numbers of Theoretical, Putatively Unique *in Silico*-Generated Tryptic Peptides of Reference Proteomes from the Eight Type Strains from the *B. cereus* Group[a]**

| strain name | species | UniProt Proteome ID | reference | last modified | putative no. of unique peptides (November 2015) |
|---|---|---|---|---|---|
| Ames ancestor | *B. an.* | UP000000594 | Ravel[75] | September 21, 2015 | 19420 |
| ATCC 14579/DSM 31/JCM 2152/NBRC 15305/NCIMB 9373/NRRL B-3711 | *B. ce.* | UP000001417 | Ivanova[76] | September 21, 2015 | 11283 |
| NVH 391−98 | *B. cy.* | UP000002300 | Lapidus[77] | September 28, 2015 | 26211 |
| ATCC 6462/DSM 2048 | *B. my.* | UP000031884 | Johnson[78] | July 27, 2015 | 11621 |
| DSM 12442 | *B. ps.* | UP000001378 | Zwick[79] | September 28, 2015 | 36179 |
| ATCC 10792/DSM 2046/CCM 19/NCIB 9134 | *B. th.* | UP000001925 | Zwick[79] | September 28, 2015 | 18864 |
| BCT-7112 | *B. to.* | UP000017860 | Jiménez[80] | September 20, 2015 | 15731 |
| WSBC 10204/DSMZ 11821 | *B. we.* | UP000030313 | Stelder[25] | September 21, 2015 | 12273 |

[a]Peptides were pre-selected according to the limits of LC−ESI MS/MS technique. Peptides were only considered if the peptides (I) lay within an *m*/*z* range of 500−6000 and (II) did not include the amino acids methionine or cysteine. *B. an.*, *B. anthracis*; *B. ce.*, *B. cereus*; *B. cy.*, *B. cytotoxicus*; *B. my.*, *B. mycoides*; *B. ps.*, *B. pseudomycoides*; *B. th.*, *B. thuringiensis*; *B. to.*, *B. toyonensis*; *B. we.*, *B. weihenstephanensis*.

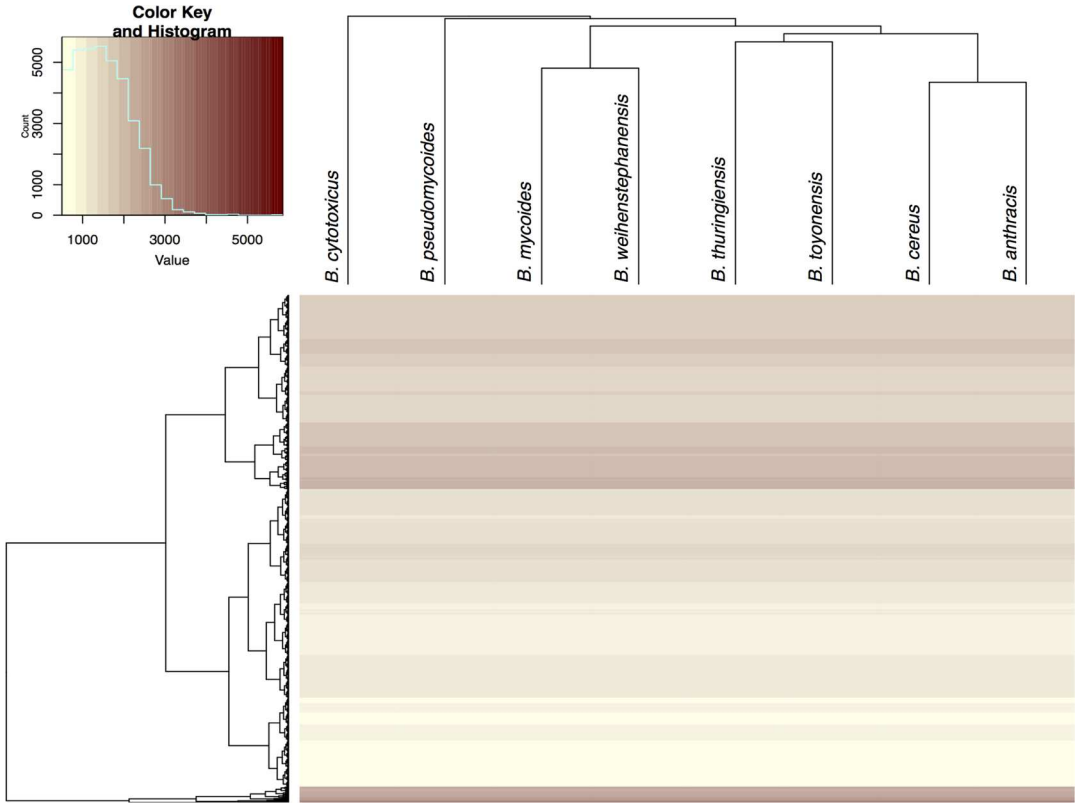


**Figure 2.** Heat map and hierarchical clustering with dendrograms for the common precursor masses within the mass range of mass spectrometers (500 Da−6000 Da) of the eight *B. cereus* group type strains. The clusterings are generated based on the accurate masses of all the peptides that have a common precursor mass (rounded to one decimal place) in all type strains. Data analysis was performed using the R language and the following packages: "cleaver",[53] "protViz",[55] "SeqInR",[54] and "gplots".[56] The Euclidean distance function and method "complete" was used in clustering, which are default settings in "heatmap.2" of the "gplots" package. The histogram on the top-left of the figure depicts the frequency of accurate masses in the data set (light blue line in the histogram, "count").

bioterrorism agents. The discovery pipeline described in this work is adaptable also to spore protein extracts. Diagnostic peptides that are exclusively expressed in spores of certain *Bacillus* species were identified by other groups.[59,60]

Alternative approaches to distinguish *B. cereus* group members on the species level have examined quantitative and qualitative differences in fatty acid (methyl esters) profiles[2,28,29] and cell wall composition profiles.[61−63] Analyses of glycosyl components of cell walls have revealed that quantitative differences even occurred among very closely related strains, for example, among several *B. anthracis* strains.[61] On the basis of findings of MLST analyses, *B. anthracis* strains were identified to form a homogeneous and clonal cluster within the *B. cereus* group.[64] Thus, cell wall components may not allow


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reliable discrimination of distinct *B. cereus* group members at the species level. Wang et al. (2015)[63] performed whole-cell profiling of surface carbohydrates using atomic force microscopy coupled with lectin probes at a single cell level of the *B. cereus* type strain (DSM 31). This technique is elusive about the quantitative detection and distribution of surface carbohydrates at the single cell level; however, it is limited to targets that have available specific binding ligands and it might be more promising if targets in addition to carbohydrates are considered. Regarding fatty acid profiles, *B. cytotoxicus* and *B. pseudomycoides* in particular contain different amounts of certain major fatty acids,[2,29,65] whereas no specific pattern or diagnostic markers could be identified among the other *B. cereus* group species.

Our data support findings from studies that have revealed limits of classical MALDI-TOF MS biotyping to discriminate species belonging to the *Bacillus* genus[45,46] (Table 3). Studies that have focused on *Bacillus* classification based on MALDI-TOF MS analysis examined strains predominantly classified as *B. anthracis*, *B. cereus*, *B. thuringiensis*, and *B. subtilis* species.[43,66−70] However, they did not investigate a selection of strains that would cover all eight species now assigned to the *B. cereus* group. We relied on an accurate database to include proteomes from *B. anthracis* when verifying the obtained candidate diagnostic peptides due to experimental limitations. We constructed a *Bacillus*-specific database composed of 113 bacilli proteomes containing 1 158 177 bacilli protein sequences downloaded from UniProt (www.uniprot.org, status May 2015). Entries on UniProt KB are either reviewed (Swiss-Prot) or unreviewed (TrEMBL). We included both databases because Swiss-Prot lacked entries of certain *B. cereus* group members (number of entries on Swiss-Prot status December 2015: *B. anthracis* (3957), *B. cereus* (4333), *B. cytotoxicus* (434), *B. mycoides* (5), *B. pseudomycoides* (0), *B. thuringiensis* (1677), *B. toyonensis* (0), and *B. weihenstephanensis* (434)). We emphasize the importance of an accurate and comprehensive database and are aware that we might have falsely excluded candidate diagnostic peptides from our final verified peptide list because of incorrect strain affiliations of entries in the TrEMBL database. Since May 2015, the addition of 58 nonredundant new proteomes predominantly from species classified as *B. cereus* demanded a second verification step by repeatedly blasting the initially verified diagnostic peptides against the entire UniProt KB (status of October 2015). We observed a remarkable decrease in the absolute number of diagnostic peptides because the query peptide sequence newly clustered with at least one protein from another *B. cereus* group species (Table 1, Table S-2). In the case of *B. weihenstephanensis*, one diagnostic peptide remained valid after the second verification. However, we assume that an increasing number of proteomes available in UniProt KB does not necessarily mean a loss of that diagnostic peptide. Taking the possible misclassification of microorganisms[9] into account, as it has been described before in case of the *B. subtilis* group and *B. cereus* group,[7,71,72] we present our results in the context of the state of art of the UniProt KB in October 2015 and the proposed review of strain affiliation suggested by Böhm and co-workers.[7] It is noteworthy that subsequent verification of diagnostic peptides is required in the dynamic process of frequent addition of proteomes to publicly available databases, but also with respect to possible reclassification of strains that have been incorrectly classified in the past. Such reclassification can lead to regaining previously excluded diagnostic peptides.

Furthermore, we reconstructed a phylogenetic tree based on theoretical precursor masses of *in silico*-generated tryptic peptides from reference proteomes of the eight *B. cereus* type-strains (Figure 2). The heat map shows that the majority of tryptic peptides that share common $m/z$ values (rounded to one decimal place) are in the range of 500 to 2500 (see top-left histogram in Figure 2). Only a few of the peptides that are shared between all eight species exhibit $m/z$ values greater than 3000. This is obvious as with longer peptide sequences the chances of introducing a different amino acid is more likely. Here, we were looking only at rounded masses that were found for all eight species. When taking the accurate $m/z$ values of *in silico*-generated tryptic peptides of the eight type-strains into account, the relatedness among these became apparent. Clustering on the *X*-axis therefore revealed a cluster comprising *B. mycoides*, *B. weihenstephanensis*, *B. thuringiensis*, *B. toyonensis*, *B. cereus*, and *B. anthracis* which share more common accurate $m/z$ values among each other as compared to the remaining species *B. cytotoxicus* and *B. pseudomycoides*.

The phylogenetic tree shows a tree topology similar to the phylogenetic relationships published by Böhm et al.[7] In their study, they generated a phylogenetic master tree from 142 *B. cereus sensu lato* strains identifying seven major phylogenetic clusters as it has been reported previously.[73,74] Group I, V, and VII include *B. pseudomycoides*, *B. toyonensis*, and *B. cytotoxicus*, respectively. These three species were distinguishable at species level (ANI border ≥96%), whereas the other five *B. cereus* group species were not assigned to solely one phylogenetic group.[7]

In this study, we showed that the *B. cereus* group type strain-specific diagnostic peptides can be detected using LC−MS/MS. The workflow described can be easily adapted to other microorganisms. It will allow the development of a diagnostic tool to rapidly and confidently identify bacteria at the level of species, subspecies, or strains based on targeted monitoring of diagnostic peptides present in the trypsin-digested protein extracts. A high-resolution and high mass accuracy selected reaction monitoring (SRM) assay is the method of choice for such analyses that will constitute an attractive and cost-effective alternative or a complement to phenotypic or genotypic methods in the future.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.6b00216.

*Bacillus cereus* group type strain-specific diagnostic peptides (PDF)

Bacillus-specific database; all proteins and peptides identified within three independent replicates of *B. cereus* type strains (with the exception of *B. anthracis* type strain) (XLSX)

*In silico*-generated tryptic peptides of reference proteomes from *B. cereus* group type strains (XLSX)

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### Author Contributions

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### Notes

The authors declare no competing financial interest.

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## ■ ABBREVIATIONS

MS, mass spectrometry; MALDI-TOF MS, matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry; LC-ESI MS/MS, liquid chromatography electrospray ionization tandem mass spectrometry; MLST, multilocus sequence typing; AFLP, amplified fragment length polymorphism; HIFU, high-intensity focused ultrasound; UniProt, Universal Protein Resource; UniProtKB, Universal Protein Resource Knowledgebase; DSMZ, German Collection of Microorganisms and Cell Cultures; EtOH, ethanol; FA, formic acid; ACN, acetonitrile; MSP, Main Spectra; HCl, hydrochloric acid; NaOH, sodium hydroxide; HCD, higher-energy collisional dissociation; MGF, Mascot generic file; FDR, false discovery rate; TIGR, The Institute for Genomic Research; ANI, average nucleotide identity

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